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Transmission of the 263K scrapie strain by the dental route

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Apart from a few cases of iatrogenic and familial human transmissible spongiform encephalopathies (TSEs) or prion diseases, the cause of Creutzfeldt–Jakob disease (CJD) remains unknown. In this paper we investigated the possibility that dental procedures may represent a potential route of infection. This was assessed by using the experimental model of scrapie in hamster. In the first part of this study we found that after intraperitoneal inoculation, oral tissues commonly involved in dental procedures (gingival and pulp tissues) bore a substantial level of infectivity. We also found high scrapie infectivity in the trigeminal ganglia, suggesting that the scrapie agent had reached the oral tissues through the sensitive terminal endings of the trigeminal nerves. In the second part of the study we inoculated a group of hamsters in the tooth pulp and showed that all of them developed scrapie disease. In these animals, we detected both infectivity and the pathological prion protein (PrPsc) in the trigeminal ganglion homolateral to the site of injection but not in the controlateral one. This finding suggests that the scrapie agent, and likely other TSE agents as well, spreads from the buccal tissues to the central nervous system through trigeminal nerves. Although these findings may not apply to humans affected by TSEs, they do raise concerns about the possible risk of transmitting these disorders through dental procedures. Particular consideration should be taken in regard to new variant CJD patients because they may harbour more infectivity in peripheral tissues than sporadic CJD patients.

Introduction

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders of humans and animals (Pocchiari, 1994). They are characterized by the accumulation of a partially protease-resistant isoform (PrPsc) of the host-encoded prion protein (PrPc) in the central nervous system (CNS) and, in some instances, other non-neural tissues of affected individuals. In humans, the natural mode of transmission is as yet unexplained; exceptions are cases of Creutzfeldt–Jakob disease (CJD) which have occurred following accidental infection after neurosurgery (Will & Matthews, 1982; el Hachimi *et al.*, 1997), implantation of EEG stereotactic electrodes (Bernoulli *et al.*, 1977), cadaveric dura mater graft or corneal transplantation (for a review see Lang *et al.*, 1998) and therapy with cadaveric pituitary-derived hormones (Brown *et al.*, 1985; Cochius *et al.*, 1990; Billette de Villemeur *et al.*, 1996; Deslys *et al.*, 1998). Epidemiological evidence (Kondo & Kuroiwa, 1982; Davanipour *et al.*, 1985; van Duijn *et al.*, 1998; Collins *et al.*, 1999) seems to exclude any correlation between tooth extraction or dental surgery and human TSEs, yet the identification of specific risk factors may be difficult through retrospective studies because the time lag between the potential iatrogenic event and the appearance of the disease may be as long as decades (Brown, 1980; Will, 1996). Moreover, small clusters of CJD cases possibly connected by dental procedures (Will & Matthews, 1982; Arakawa *et al.*, 1991) or intracranial surgery for trigeminal neuralgia (Matthews, 1975) have been reported; in experimental scrapie, the transmission of the disease has been obtained in mice by means of dental procedures (Carp, 1982) and low levels of infectivity were found in gingival tissues of intraperitoneally (i.p.) infected mice (Adams & Edgar, 1978; Carp, 1982).

The appearance of new variant CJD (nvCJD) in the UK (Will *et al.*, 1996), resulting from exposure to the agent of bovine spongiform encephalopathy (BSE; Bruce *et al.*, 1997), may lead to an increased risk, with respect to sporadic CJD, of accidental transmission from man-to-man by medical procedures, including dental work. This is because there is evidence that the level of infectivity outside the CNS is higher in nvCJD patients than in cases of the sporadic disease.

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Preliminary data have shown that tonsils from patients with nvCJD are loaded with PrPsc while those of patients with sporadic CJD are not (Hill *et al.*, 1999).

In the present paper we used scrapie-infected hamsters to measure the level of infectivity in oral tissues commonly involved in dental procedures and the efficiency of the intradental route as a way of TSE transmission.

Methods

■ **Infection of animals.** Outbred golden Syrian hamsters (purchased from Charles River, Calco, Como, Italy) were injected with the 263K strain of scrapie (Kimberlin & Walker, 1977) obtained from a 10% (w/v) PBS suspension of pooled brains from clinically affected animals. The brain suspension was centrifuged at 500 *g* for 10 min; the supernatant was collected and stored at −70 °C in 5 ml aliquots.

Before use, samples were thawed and vigorously shaken by vortexing. One group of animals was injected i.p. (0·1 ml) into the lower left quadrant of the abdomen by using sterile syringes with 26 gauge needles; another group was injected intradentally (i.d., 0·005 ml) into the lower (mandibular) left incisor. Intradental injection was performed with a Hamilton syringe on anaesthetized animals (Thiopental 0·75–0·9 mg per animal) after the incisor crown was dissected and the pulpar root cavity opened with sterile slowly rotating diamond burrs.

Animals were housed 6–12 per cage, observed for 5 days per week, and scored for the presence of clinical signs as previously described (Pocchiari *et al*, 1987).

■ **Dissection of tissues.** Intraperitoneally or intradentally scrapie-infected hamsters were sacrificed with chloroform. Brains, cervical spinal cord, trigeminal ganglia, gingival tissues and pulps of the four incisor teeth were removed by different sets of dissection instruments to minimize cross-contamination between samples. Each set was sterilized by steam autoclave at 132 °C for two cycles of 1 h. The gingival sample was taken by curetting tissue from buckle and lingual side of the frontal alveolar arch and from the palate by a scalpel blade Bard Parker 21. The pulps were removed from the apexes of extracted incisors using a K-File endodontic instrument. Samples were immediately frozen at −70 °C.

■ **Assay of infection.** Recipient hamsters (n = 8–12) were inoculated intracerebrally (i.c.) with 0·05 ml of a 10% brain, cervical spinal cord, gingival tissue, tooth pulps or trigeminal ganglia suspension. Each sample was obtained by pooling tissues from 8–10 donor animals and was homogenized with Ultraturrax in sterile PBS with different tips for each sample. Incubation periods (mean ± SEM) were measured and infectivity titres were estimated by applying these values to a dose incubation curve drawn after an end-point titration (Pocchiari *et al.*, 1989). An inverse relationship exists between dose and incubation period of the 263K strain in hamsters, which gives an average incubation period of 155·5 days for 1 LD_{50} i.c. unit in 0·05 ml of a 10% brain (and we assumed other tissues) homogenate (Pocchiari *et al.*, 1989).

Recipient hamsters were observed daily for clinical signs of scrapie disease for 400 days after i.c. infection.

■ **PrP assay.** The protease-resistant fraction of PrPsc (PrP27–30) was purified from brain and trigeminal ganglia as previously described (Xi *et al.*, 1994), electrophoresed on 15% SDS–polyacrylamide gels, and then electrotransferred to a nitrocellulose membrane. After non-specific binding was blocked with 3% fish gelatin, the membrane was processed with rabbit polyclonal antibody against hamster PrP27–30 (P8-1, 1:2000; Xi *et al.*, 1994), then processed with a goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad) and finally stained with naphtol/fast red solution.

Results

Replication of scrapie in different tissues after i.p. inoculation

After i.p. inoculation of the scrapie agent, we found infectivity both in the central and peripheral nervous system (Table 1), as well as in oral tissues. In Fig. 1 we plotted the level of scrapie infectivity at different time-points for brain, cervical spinal cord and trigeminal ganglia. The pattern of the growth curve in the brains showed a low level of infectivity titre followed by a sudden increase from the 50th day after inoculation. Two out of 10 animals injected with the 30-day-sample developed scrapie at 224 and 387 days, while none of the 10 animals inoculated with the 40-day-sample showed clinical signs of scrapie.

The growth curve of the scrapie agent in the cervical spinal cord had a pattern similar to that found in the brain, but low levels of infectivity were observed as early as 30 and 40 days after inoculation, and a small but significantly ($P < 0·001$, two-tailed Student t-test) higher titre was present in the cervical spinal cord (mean incubation period of 81·9 ± 1·3 days, n = 8) taken 60 days after inoculation than in the brain (94·0 ± 1·4 days, n = 9).

However, at the end of the clinical course the levels of infectivity of cervical spinal cord and brain were almost the same (Table 1); the mean incubation periods were 59·7 ± 1·0 days (n = 7) for animals inoculated with the cervical spinal cord and 63·5 ± 0·4 days (n = 10) for animals inoculated with

Table 1. Estimated titre (log LD_{50}/g) of tissues taken at different intervals after i.p. injection of the 263K strain of scrapie

Time after i.p. injection (days)	Tissues		
	Cervical spinal cord	Brain	Trigeminal ganglia
7	NT	NT	ND*
30	< 1	ND†	ND*
40	< 1	ND*	ND*
50	4·7	4 9	< 1
60	6·9	5·8	3·0
70	7·0	NT	NT
80	6·7	8·7	6·5
105	9·0	8·6	8·1

* No recipient animals developed scrapie disease.
† Only two out of ten recipient animals developed scrapie disease.
NT, Not tested; ND, not detectable.

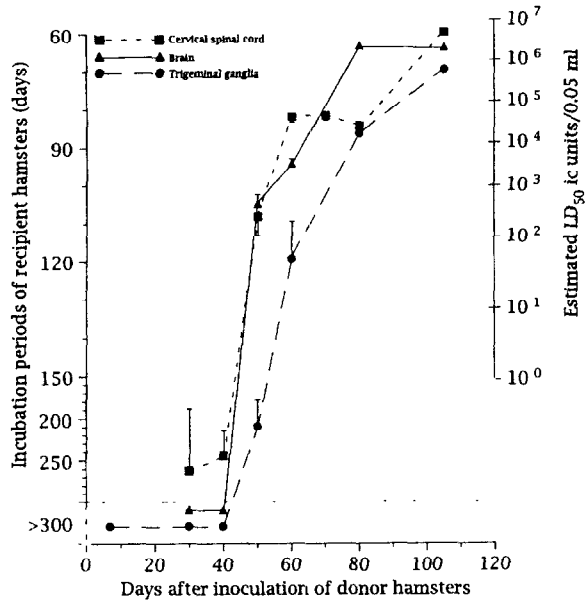

Fig. 1. Growth curve of infectivity levels measured in brain, cervical spinal cord and trigeminal ganglia of hamsters sacrificed at different time-points after intraperitoneal inoculation. Each tissue was prepared as a 10% homogenate. Bars represent the standard error of the mean.

brain material. Though delayed in timing, the growth curve of the scrapie agent in trigeminal ganglia followed the same pattern as that found in the brain, with no infectivity detectable before the 50th day and a sudden increase in the second half of the incubation period. Recipient animals inoculated with trigeminal ganglia taken at the end of the incubation period developed scrapie disease with a mean incubation period of $69{\cdot}5 \pm 1{\cdot}4$ days ($n = 19$), which is only 6 days longer than the mean incubation period observed in hamsters receiving brain taken at the same time-point.

Finally, the levels of infectivity in the gingival tissue and pulps during the clinical stage of scrapie disease were 7·2 (estimated from the mean incubation period of recipient animals, $79{\cdot}0 \pm 0$ days, $n = 7$) and 5·6 ($96{\cdot}9 \pm 0{\cdot}9$ days, $n = 10$) log LD_{50} i.c. units/g of tissue, respectively. Although these values were clearly lower than those found in the trigeminal ganglia, they were nonetheless higher than expected and clearly showed that oral tissues may harbour a good amount of infectivity. It remains, however, to be clarified why gingival tissue had a higher level of infectivity than the pulp.

Presence of PrP27–30 in a nervous peripheral station

Aliquots (less than 0·5 ml) of the homogenized trigeminal ganglia taken either 7 days after inoculation or at the end of the clinical course were used to detect PrP27–30. In accordance with the results obtained in the measurement of infectivity, no PrP27–30 was found in the 7-day-sample, while a great

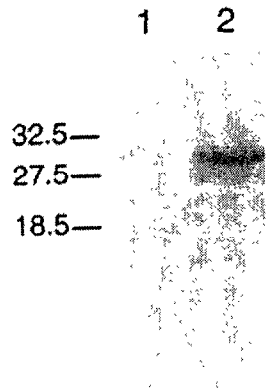

Fig. 2. Western blot detection of PrP27–30 in trigeminal ganglia of hamsters sacrificed 7 days (lane 1) or 105 days (lane 2) after intraperitoneal inoculation. Numbers on the left indicate molecular mass in kDa.

amount was seen in the 105-day-sample (Fig. 2). This result adds further evidence to the role played by the peripheral neural cells in supporting replication of the scrapie agent.

Spread of infectivity after intradental inoculation

This second set of experiments was done to assess if the dental route of infection may represent a port of entry of the scrapie agent and to explore its possible neural distribution along the trigeminal pathway. Each of the six i.d. inoculated animals developed scrapie disease (mean incubation period $152{\cdot}6 \pm 16{\cdot}0$ days), providing evidence of the efficiency of this route in the establishment of infection. To demonstrate the neural progression of the scrapie agent, left (homolateral to the injection site) and right (controlateral to the injection site) trigeminal ganglia were taken 30 days after intradental inoculation and the level of infectivity measured by i.c. inoculation into recipient hamsters. Animals receiving left trigeminal ganglia developed the disease in $281{\cdot}8 \pm 21{\cdot}9$ days ($n = 10$), while those infected with the right trigeminal ganglia did not show any clinical signs of scrapie and were sacrificed 395 days after inoculation. This result was further confirmed by detecting PrP27–30 only in the brains of animals injected with the left trigeminal ganglia (data not shown).

Discussion

In this study we found that gingival and pulp tissues of experimentally scrapie-affected hamsters bear a significant level of infectivity. These findings extend previous data of infectivity in gingival tissues of mice infected with the ME7 (Adams & Edgar, 1978) or 139A (Carp, 1982) strain of scrapie, and reinforce the notion that oral tissues in scrapie-affected rodents harvest some infectivity. It is likely that the scrapie agent reaches the buccal tissues through the sensitive terminal

endings of trigeminal branches. That is supported by the high level of scrapie infectivity (estimated titre, 8·1 log LD_{50}/g) and the great amount of PrP27–30 in the trigeminal ganglia. These results fit with other data reporting infectivity or PrPsc deposit in trigeminal ganglia of cattle affected by BSE (Wells *et al.*, 1998) and patients with CJD (Guiroy *et al.*, 1989). Altogether these data suggest that similarly to spinal ganglia and other peripheral nervous tissues (Hadlow *et al.*, 1980, 1982; Kimberlin *et al.*, 1983*a*; Groschup *et al.*, 1996; Wells *et al.*, 1998; McBride & Beekes, 1999), trigeminal ganglia can sustain the replication of scrapie and other TSE agents, and as a consequence of that, oral tissues in other TSE-susceptible species, including humans, may harbour some infectivity during the clinical course of the disease.

After i.p. inoculation, the onset of scrapie replication in the trigeminal ganglia occurs about 10 days later than in the brain and 20 days later than in the cervical spinal cord, suggesting a centrifugal spread of the scrapie agent from the sensory nuclei of the trigeminal system [extended from the medulla oblongata up to the mesencephalon and, in i.p. CJD-infected mice, showing early PrPsc deposits (Muramoto *et al.* 1993)] to the trigeminal ganglia. These findings support the view that after intraperitoneal inoculation the scrapie agent moves first centripetally to the CNS (Kimberlin & Walker, 1979, 1982; Baldauf *et al.*, 1997) and then centrifugally to the peripheral nervous system (Kimberlin *et al.*, 1983*a*), including trigeminal ganglia.

The other important result obtained by this study is that the injection of scrapie into the tooth pulp of Syrian hamsters is an efficient route of infection which gives a mean incubation period ranging between those observed after intraocular (about 130 days; Buyukmihci *et al.*, 1983; Kimberlin & Walker, 1986) and intrasciatic (about 180–190 days; Kimberlin & Walker, 1986) inoculations. Temporal differences are probably due to the relative distances between the injection sites and the brain, rather than to different efficiencies of these peripheral routes. The rate of spread of scrapie infection to the CNS, as measured following intraperitoneal inoculation (Kimberlin & Walker, 1979, 1982; Kimberlin *et al.*, 1983*a*), oral administration (Beekes *et al.*, 1996) or injection of sciatic (Kimberlin *et al.*, 1983*b*) or optic (Kimberlin & Walker, 1986; Scott & Fraser, 1989) nerves, is always equivalent to about 1 mm/day, consistent with the slowest rate of axonal transport. The same rate of scrapie propagation was estimated between teeth and trigeminal ganglia. Teeth are innervated by bipolar sensory neurons whose cell bodies are in the homolateral trigeminal ganglion. Thirty days after intrapulpal injection there was infectivity and PrP27–30 accumulation in the homolateral trigeminal ganglion but not in the controlateral one, suggesting that the infection had spread through the mandibular branch (about 25 mm long in Syrian hamsters) of the trigeminal nerve at an approximate rate of 1 mm/day.

Although these results cannot be directly applied to humans affected by TSEs, they raise the possibility that surgical instruments used during major dental procedures in CJD patients and inadequately decontaminated may represent a means of man-to-man transmission of TSEs. People incubating nvCJD are of particular concern, because they carry higher level of PrPsc, and likely infectivity, in peripheral tissues than sporadic CJD cases (Hill *et al.*, 1999). Concern will further increase if upcoming studies show that trigeminal ganglia of nvCJD patients are more affected than those of sporadic CJD cases.

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